Membrane, Device and Method for Removing Proteases from Liquids

The invention relates to a membrane for removing proteases from liquids, in particular from biological liquids and pharmaceutical solutions, which is comprised of a microporous membrane body.

The invention further relates to a device for removing proteases from liquids, in particular from biological liquids and pharmaceutical solutions, comprising a plurality of membranes connected in series.

The invention further also relates to a method for removing proteases from liquids, in particular from biological liquids and pharmaceutical solutions, by microfiltration with microporous, chemically activated membranes.

The stability of pharmaceutical solutions containing proteins is dependant on various factors and in particular on the type of the pretreatment. It is very important that various types of contamination be removed from these solutions, as regulatory authorities require mandatory numerous controls for these procedures.

For example a contamination with bacteria or fungi can be easily prevented in this manner when the solution is filtered with a sterile filtration membrane having for instance a nominal pore width of 0.2 µm. Viruses can be extracted with a chemical treatment or with the use of a strongly basic ion exchanger.

Endotoxins can be also removed with a basic ion exchanger or by means of ultrafiltration.

Proteases are enzymes which break up proteins and polypeptides. This occurs with hydrolytic splitting in adjacent amino acids which represent building blocks of the proteins. This then leads to a reduction of a formulated protein, for example an antibody, and to the occurrences of decomposition products which exert undesirable influences on patients who are treated with such a formulation.

During the processing (Down Stream Processing) of a protein, for example of an antibody manufactured with genetic technology which is produced in an animal cell culture, the antibodies can accumulate in the cell and must be released before the preprocessing from the cell into the processing medium for further processing. Intrinsic cell proteases are also released during the disintegration of the cells at same time, which can immediately break up the target protein.

In order to prevent and at least delay the effect of these proteases, it is known that small synthetic molecules can be employed which have an inhibitory effect and a very high affinity to the active center of the proteases. A disadvantage in this case is the potential danger presented by such substances, as well as their limited solubility and low stability in aqueous media. That is why a quick and efficient distribution of similar substances in large volumes is complicated.

It is further also known to an expert in this field that known chromatographic carriers, such as spherical gels, are used to immobilize suitable inhibitors. Since the removal in a desirable manner should occur as far as possible "up stream" in the purification sequence

in order to keep the production loss low, columns with a large diameter are required for the processing. This makes the step in question costly and labor intensive.

It is known from US 6,258,238 B1 that a cationic protease inhibitor can be deployed by means of bulk adsorption onto the surface of a semi-permeable membrane comprising at least an electro-negative polymer.

A disadvantage in this case is that the membrane used in this case or the used membrane body is not electrically neutral, but instead, it is negatively charged with the used monomer. It is further also difficult to find suitable inhibitors for other protease classes.

From DE 44 32 628 A1 is further also known a modularly constructed Dead-End Infiltration Unit for the selective separation of substances from fluids by filtration on porous membrane absorbers. The individual substances to be separated are kept in the filter cassettes or membranes in accordance with a specific adsorption. The adsorbed substances are selectively desorbed, eluted and absorbed with suitable elution means. No inhibitors are used according to a known procedure disclosed in DE 44 32 628 A1; wherein ion exchanging or membranes carrying ligands of pigments are employed instead. With a similar type of adsorptive application it is difficult to bind all classes of known proteases to the membrane body.

The goal of the present invention is therefore to provide membranes for the removal of a plurality of proteases from liquid, so that their effect on the liquids is prevented or at least delayed.

The removal of the proteases should take place quickly, efficiently and inexpensively. At the same time, it should be possible to remove acid proteases, metalloproteases, cystein proteases and serine proteases.

This task is achieved in connection with the characterizing clause of claim 1 so that the proteases are coupled to the membrane body with selectively binding inhibitors comprising chemically activated groups.

The advantage of the use of this type of membrane is that a higher convective flow through such a membrane is achieved in comparison to corresponding columns, because the diffusion limitation of the mass transport is negligible for practical purposes. The inhibitor amount and the surface area of the membrane required for coupling can be adjusted depending on the amount of the proteases to be removed. The membrane can be discarded after it has been used, which saves on purification and validation costs.

Acid proteases, which have an aspartic acid radical in the active center, can be adsorbed with a suitable inhibitor on the membrane body. So for example pepstatin, which inhibits efficiently pepsin, can be coupled in this manner. Metalloproteases, which have a transition metal such as zinc, in the active center, can be adsorbed for example with bestatin, diprotin or EDTA, which are coupled to the membrane body.

Cystein proteases, which have a cysteine radical in the active center, for instance papain from the papaya fruit, can be adsorbed with antipain, chymostatin or E64, which are coupled to the membrane body.

Serine proteases, which due to their ubiquitous presence are the most important family, can be also bound with suitable inhibitors, which are coupled to the membrane body.

TLCK and p-aminobenzamidine can be considered efficient inhibitors. The inhibitors mentioned above are small molecules, partially also peptide type of peptide analogs. All of them can be obtained commercially.

For cysteine proteases and serine proteases there further exist also large inhibitors of the protein type such as aprotinine, soy beans inhibitors, trypsine inhibitors or alpha-2-macroglobulin. Moreover, a great number of other inhibitors are described in literature which can be used in accordance with the invention.

The known devices have the disadvantages described above.

Another goal of the present invention is therefore to improve the known devices in such a way so as to make it possible to remove a plurality of proteases from biological liquids and pharmaceutical solutions effectively and inexpensively.

This other task is achieved according to the invention in connection with the characterizing clause of claim 10 so that the membranes are constructed according to one of the claims 1 through 9.

The device is provided with the advantages named above thanks to the construction of the membranes according to one of the claim 1 through 9. In particular, the in-series successive switching or arrangement of a plurality of membranes guarantees that the liquids to be processed will flow sequentially through all the successive membranes. The membranes can be adjusted in a relatively simple manner according to respective separation problems.

According to a preferred embodiment form of the invention, the individual membranes are provided with a membrane body that is coupled to another inhibitor.

This makes it possible to take into account the relevant protease spectrum of different liquids to be processed during the processing.

To achieve simple handling, the individual membranes are built into a suitable housing designed for a sequential through-flow.

The known methods for removal of proteases have the disadvantages named above.

That is why another goal of the present invention is to provide an efficient and inexpensive method for removing a plurality of proteases.

This task is achieved in connection with the characterizing clause of claim 13 so that inhibitors are coupled with chemically activated groups to the membranes in such a way, so that the proteases can be adsorbed and thus removed through selective binding.

Selective binding enables effective and inexpensive removing of a plurality of proteases.

Further details of the invention will become apparent from the following detailed description and from the attached drawing, illustrating examples of preferred embodiment forms of the invention.

Figure 1: A schematic illustration of a device for removing proteases.

[page 7]

A device 1 for removing proteases comprises essentially a housing 2 and four microporous membranes 3, 4, 5, 6 arranged in series.

The first membrane 3 is equipped with a first membrane body 7, to which an inhibitor binding to acidic proteases is coupled by a chemically activated group. For example pepstatin is a suitable efficient inhibitor for pepsin.

The second membrane 4 is equipped with a second membrane body8, to which an inhibitor binding to metalloproteases is coupled by a chemically activated group. Suitable inhibitors that can be considered for coupling with metalloproteases are for example bestatin, diprotin or EDTA.

The third membrane 5 is equipped with a third membrane body 9, to which an inhibitor binding to cysteine proteases is coupled by a chemically activated group. Suitable inhibitors are for example antipain, chymostatin or E 64.
The fourth membrane 6 is equipped with a fourth membrane body 10, to which an inhibitor binding to serine proteases is coupled by a chemically activated group.

Inhibitors that can be considered in this case are for example TLCK or p-aminobenzamidine.

The liquid to be processed is supplied to the first membrane 3 by a connection 11 arranged in the housing 2, wherein the corresponding acidic proteases will bind to the inhibitor of the first membrane body 7. The liquid to be processed next is then supplied to the second membrane 4, wherein the corresponding metalloproteases will be bound to the inhibitor of the second membrane body 8.

The liquid to be processed next is then supplied to the third membrane 5, wherein the corresponding cysteine proteases will bind to the inhibitor of the third membrane body 9. Finally, the liquid to be processed next is supplied to the fourth membrane 4, wherein the corresponding serine proteases will bind to the inhibitor of the fourth membrane body 10.

These provided proteases are at that point selectively removed from the processed liquid, so that the proteases can be supplied through a discharge channel 12 for further use. The membranes 3, 4, 5, 6 are discarded or disposed off with the proteases to which they are bound.

The following examples show the possibilities for binding of different inhibitors to a chemically activated membrane or membrane body, without limiting the invention in any way. The procedures are in this case carried out substantially according to the protocol described in: G. T. Hermanson, A. K. Mallia, P. K. Smith, Immobilized Affinity Ligand Techniques, Academic Press 1992, p. 119.

Example 1:

Serine protease inhibitor p-aminobenzamidine (Sigma, Deisonhofen Order No. A-7148), was dissolved in 0.05 M potassium phosphate buffer, pH 8.0, with 20 mg/ml. Ten epoxy-activated membranes with a diameter measuring 25 mm of the type 18706, made by the Sartorius AG company, were incubated overnight in this solution at 45°C. The membranes/membrane bodies were washed several times with PBS. Three membranes having a diameter of 25 mm were inserted into a filter holder (Sartorius Part No. 16517). Trypsin from a bovine pancreas (SIGMA, Order No. T-8003, Lot No. 28F-8065) was dissolved in PBS at a concentration of 1 mg/ml.

10 ml of this solution was then filtered through the membranes by using gravity. The membranes were washed with 10 ml of PBS. The bound trypsine was adjusted with 3 ml of 0.1 M glycine and eluted to pH 3.0 with HCl. The enzymatic activity of the trypsine in various fractions was determined with the synthetic substrate benzoyl arginine ethylester (BAEE), a known substrate for trypsine, in a UV spectrophotometer. These activities were then compared to the activities of trypsine solutions whose concentrations were known.

The following items were transferred with a pipette into a quartz cuvette:

0.85 ml of a 0.85 M Tris solution adjusted with HCL to pH 8.5, 0.2 ml of a solution of 2 mg/ml BAEE in water and 0.05 ml of the specimen. The increase of absorption with 253 nm took place over a period of 30 seconds.

The following results were obtained and are listed in Table 1.

Table 1: Binding of trypsine to a microporous membrane functionalized with epoxy groups

Fraction	Volume ml	Activity E253/min	µg of Trypsin Supplied	µg of Trypsin Bound
Initial Capacity	10	0.24	2,000	--
Through Flow	10	0.144		930

The test was repeated twice with the same result.

The example clearly demonstrates binding of trypsine to the membrane/membrane bodies charged with the inhibitor.

[page 10]

Example 2:

The cysteine protease inhibitor leupeptin (Sigma, Deisenhofen, Order No. L-2033) was dissolved in a 0.05 M potassium phosphate buffer, pH 8.0, at 20 mg/ml. Ten epoxy-activated membranes/membrane bodies with a diameter measuring 25 mm of the type 18706, made by the Sartorius AG Company, were incubated in this solution at 45°C overnight. The membranes were washed several times with PBS. Three membranes with a diameter of 25 mm were inserted into a filter holder (Sartorius Part No. 16517), papain from Carica papaya (Merck Art. No. 7144 Ch. 9I1 F739244, 30 000 USP – U/mg) was dissolved with 2 mg/ml in the following: 1.1 mM EDTA, 0.67 mM mercaptoethanol, 5.5 mM cysteine, 50 mM Na-acetate, pH 5.5; = complete and allowed to age for at least 30 minutes with RT. The enzymatic activity of papain in the various fractions was determined with the synthetic substrate benzoyl-arginine-nitroanilide (BANA), a known substrate for papain, in a UV spectrometer. This activity was compared to activities of papain solutions with different concentrations.

The following items were transferred with a pipette into a quartz cuvette:

0.5 ml of an enzyme solution, 0.05 ml of 25 mg/ml BANA in DMSO, 0.45 ml complete.

The following results were obtained and are listed in Table 2:

Table 2: Binding of papain to a microporous membrane functionalized with epoxy groups

Fraction	Volume Ml	Activity E253/min	µg of Trypsin Supplied	µg of Trypsin Bound
Initial Capacity	5	0.05	1,900	--
Through Flow	5	0.03		760

The test was repeated twice with the same result.

The example clearly demonstrates binding of papain to the membrane/membrane bodies charged with the inhibitor.